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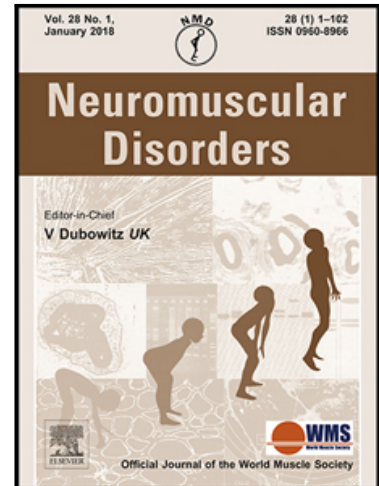
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Highlights

Description of two patients with late-onset glycogen storage disease

Characterization of two novel variants in the glycogenin-1 gene (*GYGI*)

Insights into genotype-phenotype correlations in GSD XV

Journal Pre-proof

Functional characterization of *GYGI* variants in two patients with myopathy and glycogenin-1 deficiency

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Abstract

Glycogen storage disease XV is caused by variants in the glycogenin-1 gene, *GYG1*, and presents as a predominant skeletal myopathy or cardiomyopathy.

We describe two patients with late-onset myopathy and biallelic *GYG1* variants. In patient 1, the novel c.144-2A>G splice acceptor variant and the novel frameshift variant c.631delG (p.Val211Cysfs*30) were identified, and in patient 2, the previously described c.304G>C (p.Asp102His) and c.487delG (p.Asp163Thrfs*5) variants were found. Protein analysis showed total absence of glycogenin-1 expression in patient 1, whereas in patient 2 there was reduced expression of glycogenin-1, with the residual protein being non-functional. Both patients showed glycogen and polyglucosan storage in their muscle fibers, as revealed by PAS staining and electron microscopy. Age at onset of the myopathy phenotype was 53 years and 70 years respectively, with the selective pattern of muscle involvement on MRI corroborating the pattern of weakness.

Cardiac evaluation of patient 1 and 2 did not show any specific abnormalities linked to the glycogenin-1 deficiency. In patient 2, who was shown to express the p.Asp102His mutated glycogenin-1, cardiac evaluation was still normal at age 77 years. This contrasts with the association of the p.Asp102His variant in homozygosity with a severe cardiomyopathy in several cases with an onset age between 30 and 50 years. This finding might indicate that the level of p.Asp102His mutated glycogenin-1 determines if a patient will develop a cardiomyopathy.

Key words: GYG1, polyglucosan, GSD XV, myopathy, MRI, glycogenin-1 deficiency

1. Introduction

Glycogenin-1 deficiency is a rare disorder that belongs to the group of diseases known as glycogen storage disorders (GSDs). Muscle GSDs are recessively inherited disorders of glycogen metabolism that are histopathologically characterized by storage or depletion of glycogen in muscle fibers [1, 2]. Clinically, the patients may present with exercise intolerance with muscle pain and cramps, frequently followed by myoglobinuria, or they may present with stationary, slowly progressive muscle weakness [3].

The enzymes involved in muscle glycogen synthesis (glycogenesis) include glycogen synthase (*GYS1*; GSD 0B), branching enzyme (*GBE1*; GSD IV), and glycogenin-1 (*GYGI*; GSD XV). GSD XV is inherited as a recessive trait, with compound heterozygous or homozygous variants in *GYGI*. The disease may present either as a pure adult-onset myopathy affecting adults and the elderly [2] or as a potentially severe cardiomyopathy, which may require heart transplantation [4, 5].

The *GYGI* gene encodes glycogenin-1, a core protein in muscle glycogen particles. Glycogenin-1 is a glycosyltransferase that by auto-glucosylation generates an oligosaccharide primer, covalently bound to the glycogenin. For *de novo* glycogen synthesis, glycogen synthase elongates the chain of glucose residues and branching enzyme introduces branching points to produce the soluble glycogen particles. In this way, glycogenin-1 catalyzes two auto-glucosylation reactions, a Tyr-O-glucose linkage and α 1,4-glucosidic linkages, using UDP-glucose as the donor substrate during the initiation of glycogen synthesis [6, 7].

In this paper we describe two patients with late-onset myopathy with storage of glycogen and polyglucosan due to compound heterozygous variants in *GYGI*. One patient had a heterozygous, novel splice-site variant, which by cDNA analysis was found to result in aberrant splicing, with exclusion of exon 3, a shift of the reading frame and introduction of a premature stop codon. Together with a frameshift mutation on the other allele, the result was

a total lack of glycogenin-1. The other patient had a single base deletion in one allele and a missense variant (p.Asp102His) in the other. The allele with the missense variant resulted in a non-functional glycogenin-1 protein. Patients previously reported to be homozygous for the p.Asp102His variant have developed severe cardiomyopathy with heart failure at 30-50 years of age [4], whereas our patient with late-onset myopathy had no signs or symptoms of cardiomyopathy at age 77 years.

2. Material and methods

2.1 Patients and clinical evaluation

Two unrelated Caucasian patients with a myopathy (patients 1 and 2) were investigated clinically. The study complied with the Declaration of Helsinki, and informed consent was obtained from both patients. Medical history taking and physical examination were focused on neuromuscular and cardiac symptoms and signs. Nerve conduction studies (NCS) and an EMG were performed for both patients. Cardiac function was assessed by ECG, Holter monitoring and echocardiography in both patients.

2.2 Muscle MRI

Muscle MRI studies were performed on 1.5T MRI platforms at the respective centers. Cross sections were assessed on axial T1-weighted images at pelvic, thigh, and calf levels in patient 1 and thigh level in patient 2 to evaluate the pattern of muscle involvement. Fatty replacement of muscle was graded according to the Mercuri scale. [8].

2.3 Morphological analysis

Repeat skeletal muscle biopsies were performed in patient 1. The anterior tibial muscle was biopsied at age 58 years of age and the vastus lateralis muscle at 68 years. In patient 2, a

muscle biopsy of the left vastus lateralis muscle was performed at 72 years of age. Specimens were snap-frozen in liquid propane chilled with liquid nitrogen for cryostat sectioning and histochemistry, and fixed in buffered glutaraldehyde for electron microscopy. Standard techniques were used for enzyme histochemistry, immunohistochemistry (IHC) and electron microscopy [9].

2.4 Molecular genetic analysis

Exome data of patient 1 were processed by the Genomics Platform at the Broad Institute MIT and Harvard (Boston, MA) and analyzed by the team at the John Walton Muscular Dystrophy Research Centre, Newcastle University, as described previously [10]. Population frequencies were estimated using the genome aggregation database (gnomAD), last accessed in June 2019. The Combined Annotation-Dependent Depletion (CADD) tool (version v1.4) was used as an *in silico* prediction algorithm to predict the pathogenicity of the variants identified. Variants with CADD scores greater than 20 represent the 1% highest-ranked variants genome-wide with regard to potential deleteriousness. Candidate variants in *GYGI* (NM_004130.3) were confirmed by Sanger sequencing and segregation studies were performed with available DNA samples. For functional analysis of the splice-site variant identified in *GYGI*, total RNA was isolated from frozen skeletal muscle using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). RNA was reverse transcribed with the QuantiTect reverse transcription kit (Qiagen), and *GYGI* cDNA was analyzed by PCR and Sanger sequencing.

For patient 2, DNA was studied by direct sequencing of exons and exon-intron boundaries of *GYGI* (NM_004130.3).

2.5 Western blot

Western blot was performed on protein extracted from cryostat sections of muscle biopsy specimens from both patients, with or without α -amylase treatment as previously described [4]. The supernatants (including protein) were loaded and separated on a 4–12% Bis-Tris gel (Novex; Life Technologies, Grand Island, NY), followed by electroblotting. The membrane was incubated with an antibody to the N-terminal part of human glycogenin-1 (anti-GYG1; Abnova, M07/3B5, 1:500) as the primary antibody. The SuperSignal West Dura Extended Duration Substrate (Life Technologies) was used for antibody detection.

3. Results

3.1 Clinical findings and case descriptions

A summarized overview of the clinical phenotypes of patients 1 and 2 is provided in Table 1. Patient 1, a 72-year-old man without any family history of muscle disease (Figure 1A), had had progressive complaints of myalgia and cramps from the age of 53 years. At 58 years of age, he consulted the neurology department for the first time. Clinical neurological examination at that time revealed weakness of wrist and finger extensors (MRC 4/5), tibialis anterior muscles (MRC 4/5), and extensor hallucis longus muscles (MRC 3/5), although he did not have spontaneous complaints of muscle weakness. Serum creatine kinase level was mildly increased (308 U/l) and an EMG revealed myopathic features. A muscle biopsy of the tibialis anterior muscle was performed, revealing non-specific myopathic features with type 1-fiber type predominance. On electron microscopy, subsarcolemmal glycogen accumulation was noticed in numerous fibers, as well as sarcotubular aggregates in a few fibers. After this procedure, the patient was temporarily lost to follow-up. At 60 years of age, he developed complaints of proximal muscle weakness, evident as difficulties in climbing stairs or rising from a chair. Later on, he started tripping repeatedly and noticed a decrease in hand function due to progressive weakness of the extensor muscles of the wrist. At the age of 68 years, he

consulted the neurology department again. A new muscle biopsy of the right vastus lateralis muscle was performed. Clinical examination revealed weakness (MRC 4-/5) of wrist- and finger extensors, compromising hand function, and of iliopsoas (MRC 4/5), gluteus maximus (MRC 3/5), tibialis anterior muscles (MRC 4/5), and extensor hallucis longus muscles (3/5) in the lower limbs, resulting in a waddling and steppage gait. Mild scapular winging was noted on the left side.

The patient was under regular cardiac follow-up from the age of 64 because of coronary artery disease. He had presented with angina pectoris, necessitating percutaneous coronary intervention (PCI). Urgent cardiac surgery with coronary artery bypass grafting was needed, however, due to a complication during the procedure. He did not show any signs or symptoms of an overt cardiomyopathy or major cardiac rhythm disorder during follow-up. A recent echocardiography (at the age of 70 years) revealed mild hypertrophy of the left ventricle with a normal left ventricular ejection fraction. No cardiac MRI was performed.

Patient 2, a 78-year-old man with no family history of muscle disease (Figure 2A), presented to neurological services at 72 years of age with progressive muscle weakness over a period of 2 years. The patient complained of difficulty in climbing stairs and he was unable to stand up from a squatting position without help. The patient described a mild improvement during physical activity compatible with second wind phenomenon. He did not complain of any myalgia or cramps. On clinical examination, an asymmetric pattern of proximal weakness in the upper and lower limbs was described. Muscle atrophy was found in proximal muscle groups and the patient showed a Trendelenburg gait. Abduction and elevation in the shoulder seemed to be impaired, but no winging of the scapulae was noticed. Serum CK levels were normal. There was a history of diabetes and the HbA1c levels were slightly elevated. Electroneurography did not show any neuropathy but he had a clinically mild, bilateral carpal tunnel syndrome. Electromyography on the other hand revealed a myopathic pattern. After

genetically excluding facioscapulohumeral dystrophy (FSHD), a biopsy of the left vastus lateralis muscle was performed. Cardiac monitoring was not performed regularly, but the last examination at 77 years of age showed no signs of cardiomyopathy. Holter monitoring revealed no arrhythmias. No cardiac MRI was performed.

3.2 Muscle MRI

In patient 1, muscle MRI studies at the age of 58 years revealed a myopathic pattern of selective muscle involvement. End-stage involvement of the gluteus maximus muscles was already observed at that time. At thigh level, moderate involvement of biceps femoris caput longus, semimembranosus and adductor magnus muscles was noted, with relative sparing of the semitendinosus muscle, and also mild involvement of the vastus intermedius and medialis on the right side. Calf muscles were relatively spared, with only mild involvement of soleus and medial gastrocnemius muscles (Figure 3A-D).

MRI studies of patient 1 at the age of 68 years showed end-stage involvement of vastus intermedius, biceps femoris caput longus, semimembranosus and adductor magnus muscles, and also moderate involvement of vastus medialis muscles. Of the posterior thigh muscles, the semitendinosus muscles were still relatively spared. At calf level, moderate involvement of medial and lateral gastrocnemius and the soleus and mild involvement of the tibialis anterior muscle was noted (Figure 3E-H).

In patient 2, MRI of the thigh was performed at the age of 78 years. There was end-stage involvement of gluteus maximus, adductor magnus, quadriceps femoris (with selective sparing of rectus femoris) and biceps femoris muscles (Figure 3I-J).

3.3 Muscle histopathology

In patient 1, a muscle biopsy of the right vastus lateralis muscle, performed at the age of 68 years, revealed nonspecific myopathic features with increased variability in fiber size, type 1 fiber predominance, an increased number of internalized nuclei, and periodic acid-Schiff (PAS) positive aggregates in scattered muscle fibers (Figure 1C-D). The aggregates showed immunoreactivity to desmin, α -actinin, and titin (Figure 1E-F). No muscle fiber necrosis or endomysial fibrosis was noted, but a few—presumably regenerating—fibers with immunoreactivity to neonatal myosin heavy chain were observed. Ultrastructurally, the aggregates displayed a mixed pattern of fibrillar material and apparently normal glycogen granules (Figure 1G-H).

In patient 2, a muscle biopsy of the left vastus lateralis muscle was performed at the age of 72 years. There was an increased variability in fiber size and an increased percentage of internalized nuclei was noted, as well as a variable increase in endomysial connective tissue (Figure 2C). PAS-positive aggregates were found in a large number of fibers that were otherwise depleted of glycogen. With α -amylase treatment, most but not all of the PAS-positive aggregates were digested (Figure 2D and E). There was a type-1 muscle fiber predominance, with the storage material mainly being identified in the fibers expressing slow myosin (Figure 3G). The aggregates showed strong myophosphorylase activity and they were immunoreactive for desmin and p62 (Figure 3F and H). Electron microscopy revealed that the aggregates were mainly composed of material appearing as glycogen, yet there were also regions with a predominance of fibrillar material similar to that found in patient 1.

3.4 Molecular genetic analysis

Both patients appeared to harbor compound heterozygous rare variants in *GYGI* (NM_004130.3), with *in silico* prediction algorithms being in favor of pathogenicity. For patient 1, the novel c.631delG (p.Val211Cysfs*30) frameshift variant with a CADD Score of

35 and a gnomAD allele frequency of 7.96×10^{-6} and the novel c.144-2A>G splice acceptor variant of exon 3 with a CADD Score of 33 and a gnomAD allele frequency of 0 were identified (Figure 4A and 5A). Apart from a trace of the normal sized *GYGI* transcript, analysis of *GYGI* cDNA from skeletal muscle of patient 1 revealed an aberrantly spliced transcript with skipping of exon 3, sized 175 base pairs (bp), although this allele predicted to result in a shift of the reading frame with insertion of a premature stop codon (p.Lys49Serfs*2) (Figure 4B). Patient 2 was compound heterozygous for two variants both of which have been previously reported in patients with glycogenin-1 deficiency, but not in combination: c.304G>C (p.Asp102His) and c.487delG (p.Asp163Thrfs*5) (Figure 5B) [4, 5, 11, 12].

3.5 Western blot

Protein expression of glycogenin-1 was studied by western blot analysis of skeletal muscle tissue in both patients, both with and without α -amylase treatment. In controls, glycogenin-1 was only detected by western blot analysis after treatment with α -amylase, which cleaves off the glucose residues in the glycogen particles and releases the glycogenin-1 protein that is located in the core of glycogen particles (Figure 1B and 2B). In patient 1, glycogenin-1 could not be detected either with or without α -amylase treatment, demonstrating complete absence of the glycogenin-1 protein (Figure 1B). In patient 2, glycogenin-1 was detected both with and without α -amylase treatment, showing that it was not glucosylated. Overall, the level of this apparently non-functional glycogenin-1 was lower than control samples (Figure 2B).

4. Discussion

We have described two patients with GSD XV due to compound heterozygous variants in the *GYGI* gene and provide potential new insights in genotype-phenotype correlations of this rare disorder.

The two novel variants identified in patient 1, c.144-2A>G and c.631delG, were shown to exert a loss-of-function (LOF) effect, evident by the complete loss of the glycogenin-1 protein. At RNA-level, the c.144-2A>G variant was proven to cause aberrant splicing with skipping of the 175 bp long exon 3, resulting in a shift of the reading frame and a premature stop codon. Small amounts of this aberrant transcript, as well as the wild type transcript were however identified in the patient's muscle by qualitative PCR experiments. This most likely implies that transcripts to a very limited extent are not immediately fully targeted by nonsense-mediated mRNA decay (NMD). Quantitatively however, levels of these transcripts are biologically irrelevant considering the western blot findings showing complete loss of glycogenin-1.

In patient 2, two previously described variants, c.304G>C (p.Asp102His) and c.487delG (p.Asp163Thrfs*5), were identified. Protein analysis revealed a reduced amount of glycogenin-1 protein that was not able to perform the auto-glucosylation reactions required for normal glycogen synthesis.

The two patients add to a number of previously described cases with GSD XV due to inactivation and/or reduction of normal glycogenin-1 protein. Strikingly, two distinct phenotypes appear to exist of this disease, with only minor overlap. The most common presentation is a late onset, apparently pure skeletal muscle myopathy with muscle weakness as the main manifestation. The alternative presentation on the other side of the spectrum is a severe young adult onset cardiomyopathy with only minor skeletal muscle involvement [2, 4]. Pathomechanisms underlying this strict separation of phenotypes are currently unknown, but

most patients so far described with a severe cardiomyopathy unmistakably directly linked to the *GYGI*-related disease harbored the p.Asp102His missense variant in homozygosity, and expressed mutated glycogenin-1 in cardiac muscle [4]. One other GSD XV patient showing a myopathy phenotype also showed marked cardiac involvement which is probably also primarily related to the *GYGI*-related disease, yet of a different nature [5]. This patient, manifesting an early adult onset cardiac arrhythmia phenotype, harbored the p.Thr83Met missense variant in compound heterozygosity with a variant resulting in frameshift and a premature stop codon, and was shown to express mutated glycogenin-1 in both skeletal and cardiac muscle. Ejection fraction on echocardiography was normal, yet large vacuoles of PAS-positive material were visualized in cardiomyocytes, similarly as described for the three patients harboring the homozygous p.Asp102His variant [4]. Most other currently reported GSD XV patients have normal cardiac assessments, but a few show apparently coincidental cardiac abnormalities due to concomitant coronary artery disease (as in our patient 1) or hypertension. Only two of these patients, showing a late-onset myopathy, harbor a homozygous missense variant in *GYGI*, p.Alal6Pro and p.His212Tyr respectively, other harbor LOF function variants [12, 15].

Most patients with a pure skeletal myopathy demonstrated absence of—or severe deficiency in—glycogenin-1 protein by western blotting. It is therefore of interest that our patient 2 is the first known glycogenin-1 deficient patient to be heterozygous for a specific missense variant that has previously been identified in homozygous form in several patients with a severe cardiomyopathy [4]. The mutated residue, p.Asp102His, is located in the catalytically active site of the glycogenin-1 molecule and completely abolishes its auto-glucosylation, as demonstrated by protein analyses in tissue from affected individuals and by functional *in vitro* studies [4]. Patient 2 did not show any sign of cardiomyopathy at age 77 years in spite of a severe myopathy. It may be speculated that this mutated protein, in addition to being unable

to prime normal glycogen synthesis, also exhibits a toxic (gain-of-function) effect in the heart when expressed at sufficient levels as seen in patients who are homozygous for the same variant. Additional experiments are needed to support this hypothesis.

The clinical presentation of myopathy in patients with glycogenin-1 deficiency (GSD XV) is variable and may include both distal and proximal muscle weakness, myalgia and also exercise intolerance. Some patients have mainly upper limb involvement whereas others have involvement mainly of gluteal muscles, and muscles of lower limbs. Several cases have presented with asymmetric muscle weakness and wasting. In accordance with these observations, the muscle MRI findings have been variable, but have often shown a selective pattern of muscle involvement [11, 13-16]. However, as in the two patients reported here, the gluteal, quadriceps, and adductor magnus muscles frequently show severe selective involvement [11, 13, 14].

No systematic studies on protein expression in the PAS-positive muscle fiber aggregates have been performed, but ubiquitin and p62 have been reported in several studies, indicating that proteins in the aggregates are marked for proteasomal or lysosomal degradation. However, the marked accumulation of material indicates a failure of the system to degrade it. This is a common finding also in other muscle glycogen storage disorders with polyglucosan, such as in RBCK1 deficiency [17], and is probably related to the nature of the storage material rather than to a primary defect of the lysosomal or proteasomal degradation pathways.

Although most of the GSD XV storage material can be digested by α -amylase as seen in our patients 1 and 2, there is some residual PAS-positive storage material, and the ultrastructural appearance of the material is partly characteristic of polyglucosan with a fibrillar structure. Thus, the storage material in glycogenin-1 deficiency is different from what is found in branching enzyme and RBCK1 deficiency by being less α -amylase resistant—disorders that

are otherwise morphologically similar [2]. This indicates that the composition of the aggregates differs between different polyglucosan storage disorders, which is indeed expected regarding the pathomechanistic differences. Further comparative analysis of the storage material in these different disorders may shed some light on their pathogenesis.

In muscle of patient 2, showing numerous fibers containing PAS-positive aggregates, marked type-1 fiber predominance was noted, with aggregates being restricted to type-1 fibers. This pattern of glycogen storage—mainly in type-1 fibers, which has also been described in other patients with glycogenin-1 deficiency [15]—is surprising, since there is usually more glycogen in the glycolytic type-2 fibers than in oxidative type-1 fibers. A high level of glycogen metabolism is therefore probably not a factor in increasing the susceptibility of muscle fibers to storage of abnormal glycogen when there is glycogenin-1 deficiency. Another possibility could be that a defect in glycogen metabolism in muscle fibers with polyglucosan storage induces a metabolic shift and transformation of fibers from type-2 to type-1. This would explain the type-1 fiber predominance seen in patient 2 and in the patient described by Luo et al. [15]. Impaired glycogen metabolism, as in glycogen synthase deficiency causes such a metabolic adaptation with a marked type-1 fiber predominance [18].

In patient 1 the abnormal storage material was found in fibers also showing normal glycogen in the rest of these fibers, whereas in patient 2 the fibers with inclusions were otherwise depleted of glycogen. This apparent difference may be a sampling effect since the pattern varies between patients and also within the same muscle of patients [4, 12, 15].

As shown in this and other studies glycogenin-1 appears to be dispensable for glycogen synthesis in muscle [19]. There have been speculations about an alternative primer for

glycogen synthesis but results from investigations on glycogenin knockout mice, in which the glycogen content was found to be even higher than in controls, indicate that glycogen synthesis could occur without a protein primer such as glycogenin [20].

In conclusion, we report two patients with GSD XV. Both patients were found to be compound heterozygous for *GYGI* variants, of which two were novel, and presented with a characteristic late-onset myopathy without any apparent cardiomyopathy. One patient showed complete absence of glycogenin-1 protein whereas the other showed some expression of mutated glycogenin-1. This patient was heterozygous for a missense variant that is associated with a severe cardiomyopathy in homozygous individuals, an observation that may be a clue towards pathomechanisms of glycogenin-1-associated cardiomyopathy, which requires further research.

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Conflicts of interests

The authors have no financial conflicts of interests.

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Figure legends

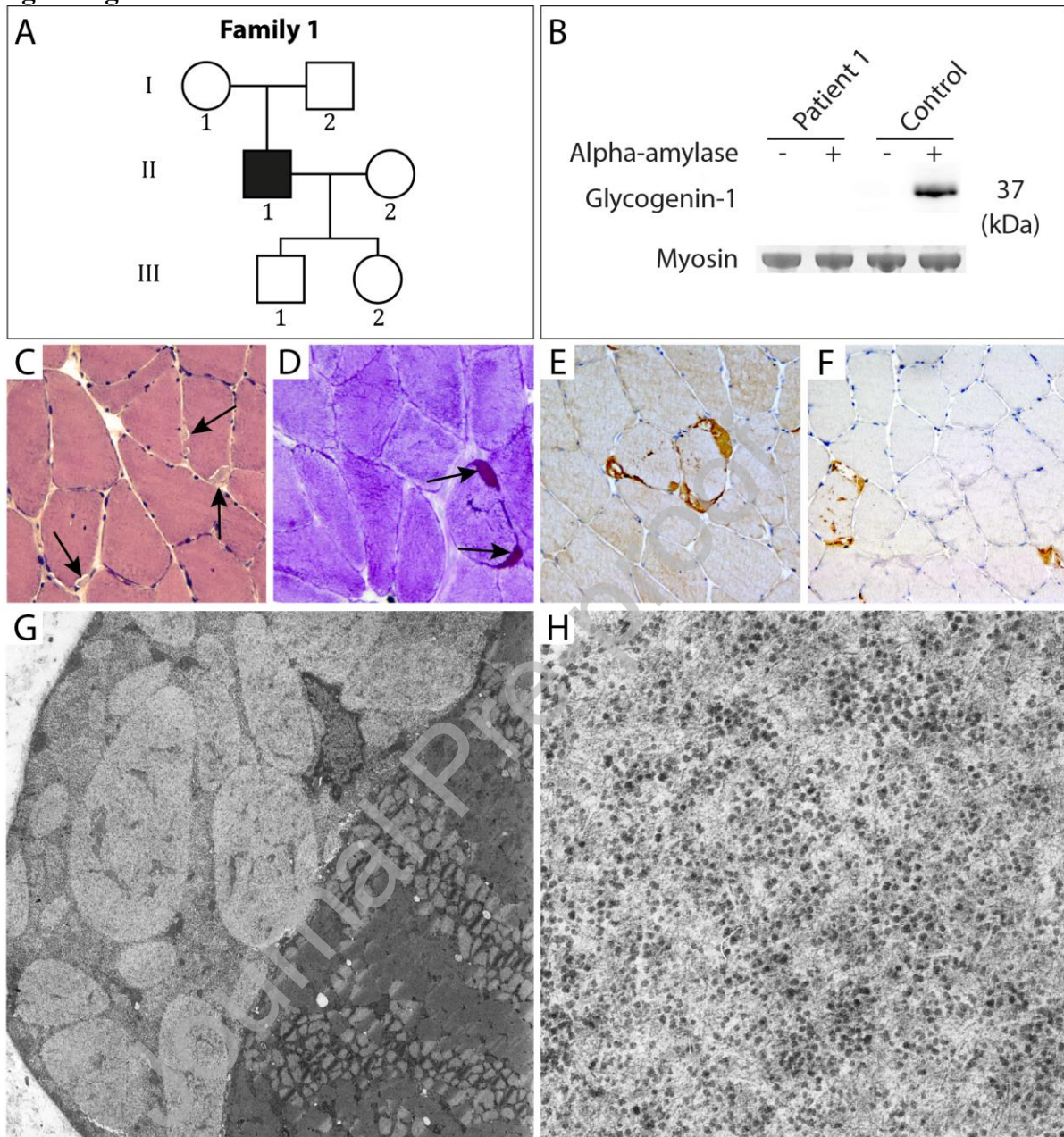


Figure 1. Patient 1. (A) Pedigree of the family. (B) Western blot analysis of glycogenin-1 using protein extracted from a skeletal muscle biopsy specimen from the patient and from a control sample, performed without (-) or with alpha-amylase (+) treatment. In the patient sample, glycogenin-1 was not detected irrespective of whether or not α -amylase treatment was used whereas in an aged-matched control sample glycogenin-1 was detected, but only after α -amylase treatment. The band corresponding to myosin heavy chain was used as

loading control. (C-H) Muscle histopathology. (C) The muscle fibers showed variation in size, with some internal nuclei and pale-staining aggregates in a few fibers (arrows). (D) The aggregates were strongly PAS-positive (arrows). (E) The aggregates were immunoreactive for desmin. (F) Aggregates immunoreactive for α -actinin. (G) Electron micrograph demonstrating compartmentalization of the storage material. (H) At high magnification the ultrastructural appearance of the storage material showed a mixture of fibrillar material and glycogen granules.

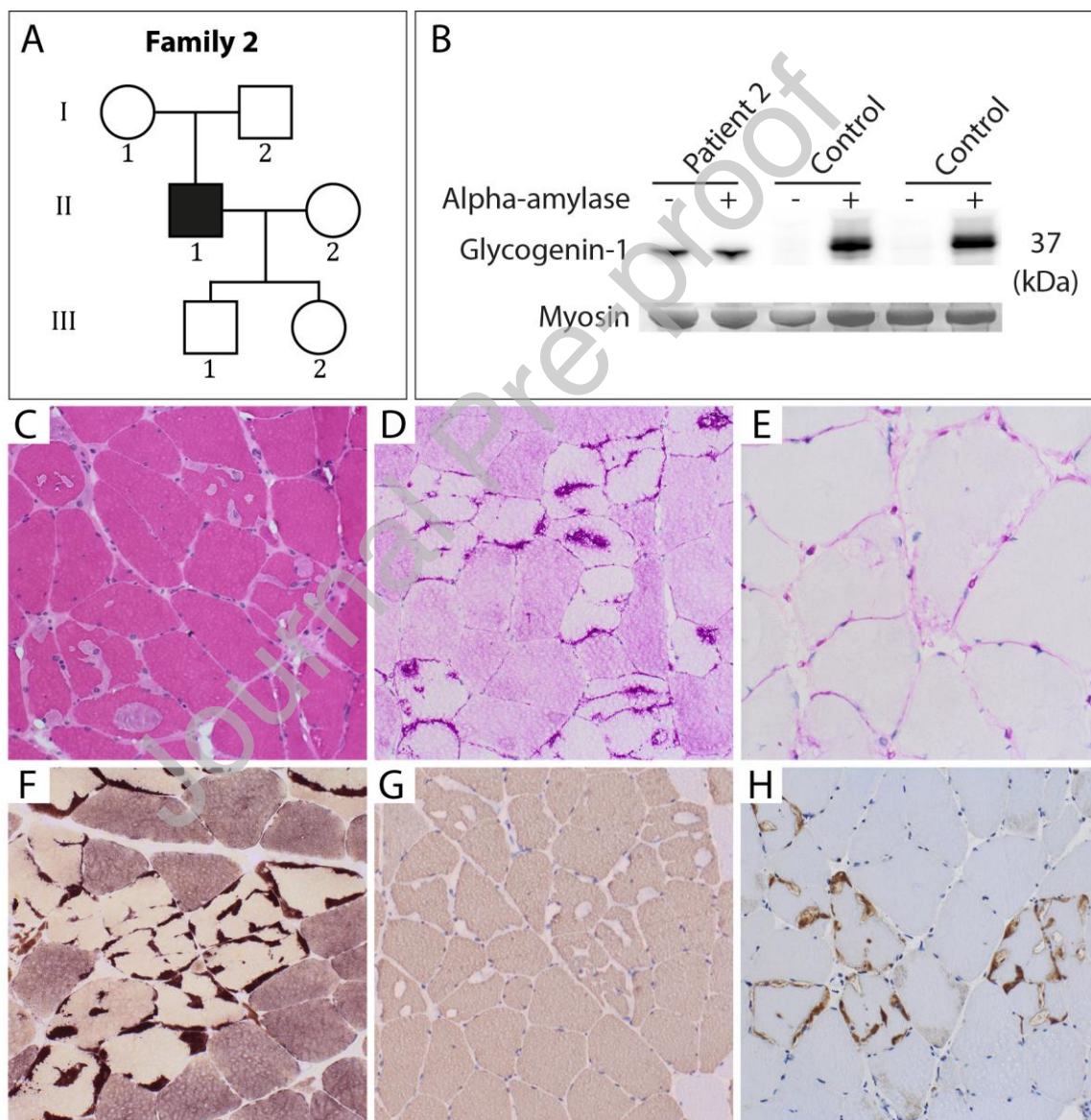


Figure 2. Patient 2. (A) Pedigree of the family. (B) Western blot analysis of glycogenin-1 using protein extracted from skeletal muscle biopsy specimens from the patient and from

control samples, performed without (-) or with α -amylase (+) treatment. In the patient, glycogenin-1 was detected both without and with α -amylase treatment, in contrast to aged-matched control samples where glycogenin-1 was only detected after α -amylase treatment. The band corresponding to myosin heavy chain was used as loading control. (C-H) Muscle histopathology. (C) Numerous fibers showed pale-staining aggregates (H&E). (D) The aggregates were strongly PAS-positive and located in fibers otherwise depleted of normal glycogen (PAS). (E) Sections treated with α -amylase showed almost complete loss of PAS-positive storage material (PAS-diastrase). (F) The myophosphorylase activity followed the pattern of PAS positivity as seen in Figure 3D (myophosphorylase enzyme histochemistry) (G) Immunohistochemistry of slow myosin heavy chain demonstrating type-1 fiber predominance. Inclusions were found in type-1 fibers. (H) Immunohistochemistry for p62 (sequestosome 1) showed positive staining restricted to aggregates of PAS-positive material.

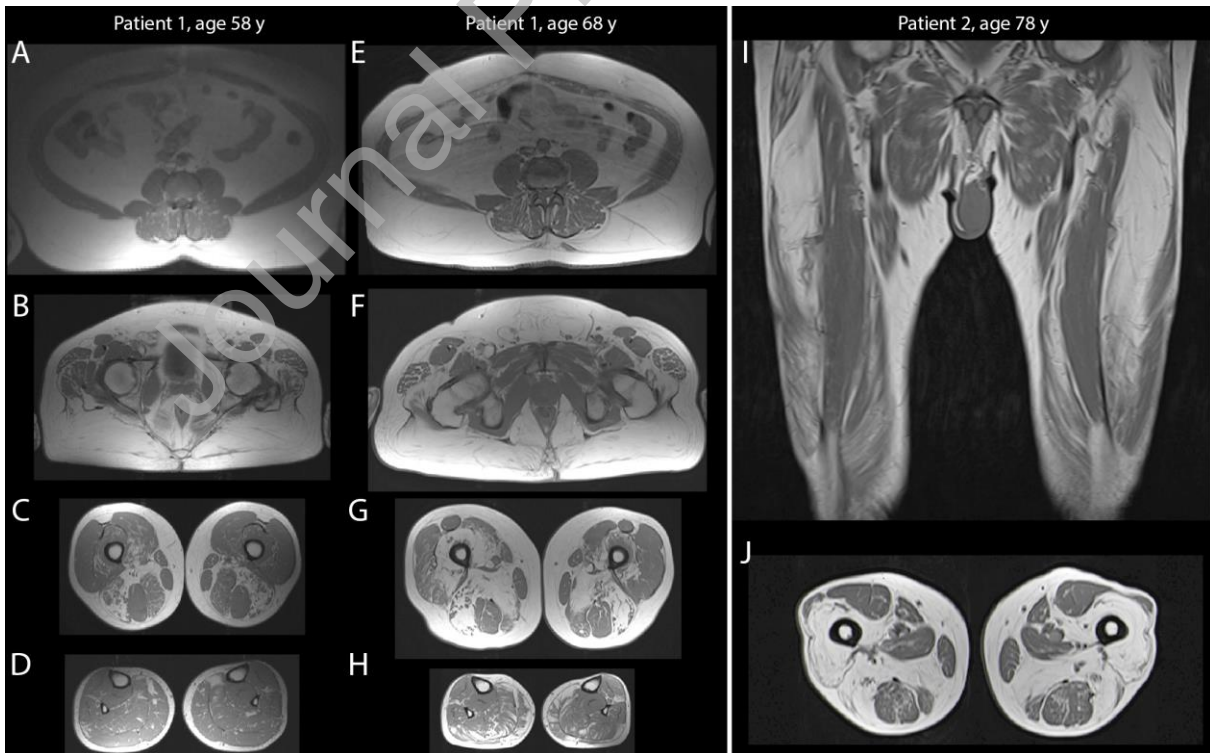


Figure 3. Muscle MRI findings in patients 1 and 2. Axial T1-weighted images from repeated MRI studies are shown for patient 1, performed at 58 years of age (A-D) and 68 (E-H) years

respectively. End-stage involvement of gluteus maximus muscles was already noted at the age of 58 years. Over a period of 10 years' time, progressive involvement of thigh muscles was noted, with most severe involvement of the vastus intermedius, biceps femoris (caput longus), adductor magnus and semimembranosus muscles. Calf muscles (mainly anterior tibial, soleus and gastrocnemius muscles), initially being relatively spared, showed mild involvement at the age 68 years of age. (I-J) MRI images for patient 2 at 78 years of age. (I) A representative coronal view showing the spared rectus femoris. (J) Axial T1-weighted images at thigh level show end-stage involvement of vastus intermedius, lateralis and medialis (with selective sparing of the rectus femoris), as well as of adductor magnus and biceps femoris muscles.

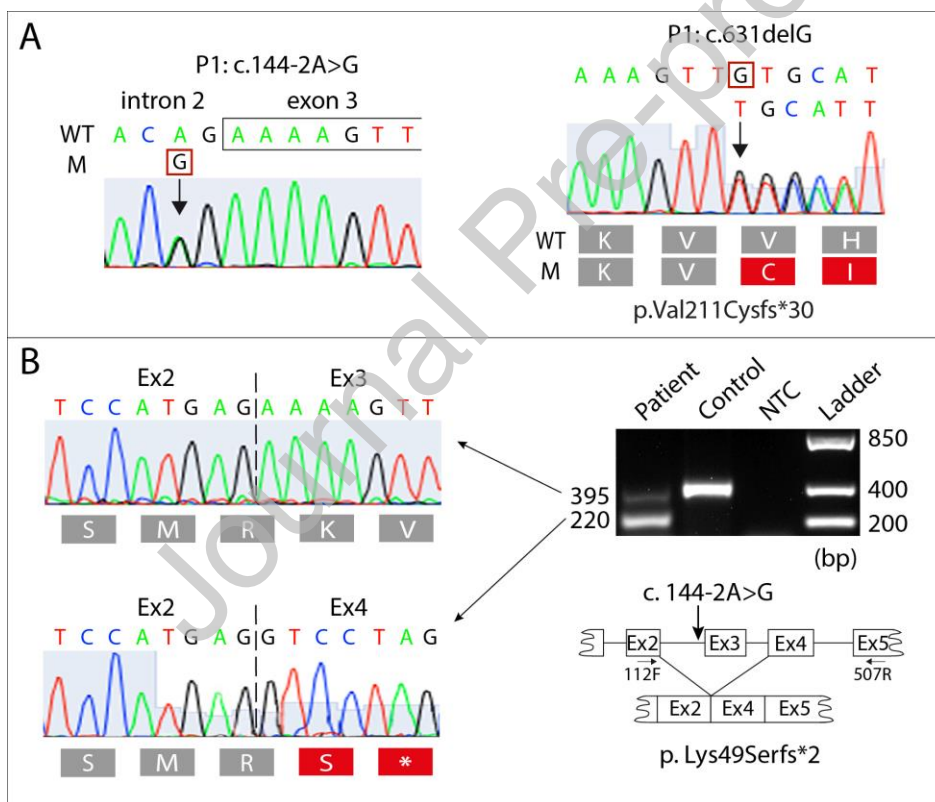


Figure 4. *GYGI* analysis in patient 1. (A) Sequencing chromatograms demonstrating the two novel heterozygous variants identified in patient 1 on genomic DNA, c.144-2A>G, which would abolish normal splicing, and the single base deletion c.631delG, which would result in shift of the reading frame and a premature stop codon (p.Val211Cysfs*30). (B) Agarose gel

of a *GYGI* PCR product of an amplicon sized 395 base pairs (bp) from cDNA from control and patient 1. The c.144-2A>G variant was shown to result in aberrant splicing, with transcript of different sizes being detected in muscle of patient 1. The shortest transcript represents a transcript in which the 175 bp long exon 3 is skipped. Although this out-of-frame deletion is predicted to result in a premature stop codon (p.Lys49Serfs*2), still a small amount of this transcript was amplified. Additionally, an even smaller amount of a transcript with a similar length as in control muscle was still identified because of not immediately fully targeted by NMD.

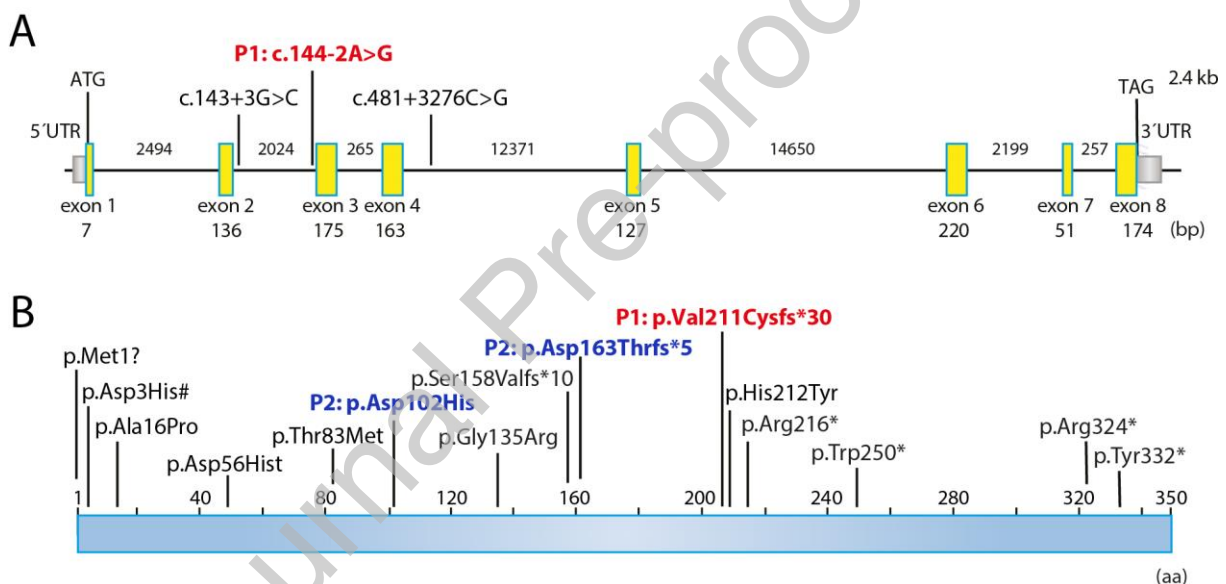


Figure 5. Overview of novel and previously reported *GYGI* variants. (A) Schematic illustration of *GYGI* (NM_004130.3), at DNA-level, showing pathogenic intronic variants. (B) Schematic illustration of *GYGI* at the protein level, showing pathogenic missense, frameshift and nonsense variants. Variants identified in this study have been marked in red (patient 1 with two novel variants) and blue (patient 2 with two previously described variants). p.Met1?, start lost variant. #, variant affecting splicing.

Table 1. Clinical findings.

			Patient 1	Patient 2
Gender			Male	Male
Ethnicity			Caucasian	Caucasian
Age at onset, y			53	70
Presenting symptoms			Myalgia, cramps	Muscle weakness
Exercise intolerance, myalgia			Yes	No
Palpitations			No	No
Syncopal episodes			No	No
Age at last examination, y			70	72 (follow-up at 77)
Weakness	Proximal	UL	No	Yes
		LL	Yes	Yes
	Distal	UL	Yes	No
		LL	Yes	No
Skeletal muscle atrophy			Thenar	Proximal muscles LL and UL
Calf hypertrophy			Yes	No
Scapular winging			Medial winging on the left	No
Ambulation status			Ambulant	Ambulant
Serum CK (U/l)			453	Normal
EMG (age, y)			Myopathic (58)	Myopathic (72)
Coronary artery disease			Yes	No
Resting ECG			Normal	Normal
Echocardiography			Mild hypertrophy of the left ventricle	Normal
Holter monitoring			Frequent VES	Normal
Biopsied muscle (age, y)			Anterior tibial muscle (58) Lateral vastus (68)	Left vastus lateralis muscle (72)

Abbreviations: LL, lower limbs; UL, upper limbs; VES, ventricular extra-systoles.